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We propose that the expression of several unknown genes is affected by gamma radiation. Abnormal expression of these genes maybe one of the early steps in breast carcinogenesis induces by radiation. We plan to establish an assay that will allow us to screen for breast cells that contain a single mutation by gene trapping. We will be able o detect changes in eh expression of a gene upon treatment with different doses of radiation. These radiation-responsive genes will be identified through the rapid amplification of cDNA ends procedure and sequenced. Cells that are affected by radiation will be isolated and analyzed to see if the changes can lead to transformation of the normal breast epithelial cell into a neoplastic cell. This assay may prove to be a powerful tool in the identification of novel genes that are affected by gamma irradiation in the early stages of breast cancer progression.

The 3'RACE protocol has recently been completed and thirty one genes potential genes were sequenced. Of these, six candidate genes were found. They include: human creatine kinase gene, human androgen receptor, human DORA reverse strand protein 1 (DREV1), human eukaryotic translation elongation factor 1 beta 2 (EEF!B@), human ribosomal protein L27, and human DNA clone epithelial cells as discussed in the statement of work.

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G protein, G protein-coupled receptor, extracellular signal-regulated kinase, Lysophosphatidic acid, pertussis toxin

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INTRODUCTION

We propose that the expression of several unknown genes is affected by gamma irradiation. The subject and purpose of our research is that the abnormal expression of these genes may be one of the early steps in breast carcinogenesis induced by radiation. We plan to establish an assay that will allow us to screen for breast cells that contain a single mutation by gene trapping. We will be able to detect changes in the expression of a gene upon treatment with different doses of radiation. These radiation-responsive genes will be identified and analyzed to see if the changes can lead to transformation of the normal breast epithelial cell into a neoplastic cell. This assay may prove to be a powerful tool in the identification of novel genes that are affected by gamma irradiation in the early stages of breast cancer progression.

BODY

RESEARCH TRAINING

Ongoing training is very important throughout my predoctoral period. My department, Environmental and Radiological Health Sciences, places an important focus on training. Weekly, I attend Advanced Radiation Biology journal meetings where faculty and students interact and discuss current and relevant papers in breast cancer research and radiation effects. Each attendee presents one journal article every semester and leads the discussion. Weekly there is a Cell and Molecular Biology seminar where invited visiting speakers give a 50 minute presentation and discussion about relevant topics such as breast cancer research, cell signaling, and many more. There are also two departmental seminars I attend weekly where visiting speakers, as well as graduate students present their research. It is very important for my training that I keep current with the latest research techniques and discoveries by attending these meetings. My mentor, Dr. Robert Ullrich, is currently the Oncology Chair of the Veterinary Teaching Hospital here on campus, so I am also exposed to more clinical cancer research seminars and meetings that I attend there as well.

On July 11th through the 14th I attended the 94th annual meeting of the American Association for Cancer Research meeting in Washington D. C. This contributed a great deal to my overall predoctoral training by exposing me to cancer research scientists from all over the world. I was able to attend numerous oral and poster presentations and learn about the latest advances being made in breast cancer and radiation research.

RESEARCH PURPOSE & GOALS

We plan to identify novel genes affected by gamma irradiation and to characterize their function using a gene-trapped library of human mammary epithelial cells. We hypothesize that the mutation of these novel genes or its abnormal expression is one of the causes of early breast carcinogenesis. Mounting evidence suggests that gene products may function differently depending on cell type, developmental stage, or species. Thus, to identify novel gene(s) critical for the initiation of breast cancer, we need to study the irradiation effects of "loss of function" of a gene product in human breast epithelial cells.

The issue of how low dose gamma radiation may lead to breast cancer will be addressed by studying the genes affected by low dose gamma irradiation. We will focus on the trapped genes whose expression are immediately changed by a single dose of gamma irradiation, determine if this is a dose-dependent effect and further analyze whether this effect can lead to transformation of the breast cells.

The following are specific aims as outlined in the approved statement of work:

Specific Aim 1: To establish a high throughput assay for detection of variation in gene expression in human mammary epithelial cells using gene-trapped MCF10A clones.
Specific Aim 2: To determine the effect of gamma irradiation on expression of reporter protein GFP (green fluorescent protein).
Specific Aim 3: To characterize the effect of gamma irradiation on transformation of human mammary epithelial cells.
Specific Aim 4: To identify the trapped genes affected by gamma irradiation.

RESEARCH PROGRESS

Currently, specific aim 1 and specific aim 2 are completed. Attached in the appendices are color representations of the completed construction of the gene-trapped MCF10A clonal library as seen under a fluorescent microscope. This is included in one of my PowerPoint presentations. It is clearly observed in the pictorials, that the bright green fluorescence luminating from the cells is due to the retrovirus pRET being incorporated into the genome.

A total of 192 gene-trapped clones were analyzed by the construction of a single cell assay in 96-well plates. This was done to obtain single cell clones, hopefully each representing a different trapped gene. One 96-well plate contained the pooled gene-trapped MCF10A cells sorted by flow cytometry into a GFP positive pool and the other 96-well plate contained the pooled gene-trapped MCF10A cells sorted by flow cytometry into a GFP negative pool. Graphical representations of the flow cytometry data are included in the appendices in one of my power point presentations. Replica plating was then done from both of the original single cell assay plates for the following GFP expression levels to be measured at: basal, control, master, store at -80°C, 0.5 Gy, and 2.0 Gy gamma irradiated. GFP measurements were made with a microplate reader by the way of a sandwich ELISA assay. The sandwich ELISA assay was accomplished by first expanding the 96-well plates with the single cell clones into 24-well plates. These 24-well plates were then expanded further to allow for 2 wells for each single cell assay clone. This was done so that one well could be further expanded and frozen for later use and the other well would be utilized to collect the cell lysate from for the ELISA assay. All of the 24-well plates were then irradiated with 2.0 Gy from a ^{137}Cs source. The following antibodies were used for the sandwich ELISA assay: anti-GFP (Mouse) was the primary antibody and peroxidase IgG mouse (Rabbit) was the secondary antibody. The entire protocol for the sandwich ELISA assay is included in the appendices.

Graphical representations of the gene expression of GFP after 2.0 Gy gamma radiation dose from a ^{137}Cs source is attached as well. Here, clones that were up- or down-regulated at least 2-fold from basal readings were expanded for further analysis. The basal GFP readings of the gene-trapped clones are included in the appendices for comparison to the 2.0 Gy GFP readings. Out of the 192 clones analyzed, 92 were up- or down-regulated at least 2-fold in comparison to basal GFP readings. These clones were expanded in culture and RNA was collected for gene analysis.

There was a slight change in the order of the approved statement of work next. Specific aim 4 was undertaken prior to the characterization of the effect of gamma irradiation on the transformation of the MCF10A cells. This was felt to be an important substitution due to the fact that the gene that had been trapped should be identified before transformation assays were

undertaken. Transformation assays are very tedious and time consuming. If, for example, the gene trapped was an artifact, then the process of analyzing for transformation could be skipped.

Specific aim 4 dealt with the characterization of the trapped genes that were causing either an up- or down-regulation upon treatment with 2.0 Gy. To analyze this, the gene-trapped clones were expanded and RNA was collected by using Qiagen's RNeasy kit. The protocol for this procedure is attached in the appendices. The RNA was then reverse transcribed into cDNA and amplified by the use of the Advantage-GC cDNA polymerase kit from BD Biosciences and the 3'RACE protocol from Invitrogen. Both protocols are included in the appendices. Gene specific primers for the neomycin marker found on our pRET retrovirus and against the polyA tail of the endogenous gene were designed. The sequences of the primers are given in the appendices. After each step, reverse transcription, first strand cDNA synthesis, and second strand cDNA synthesis, agarose gels were run to verify that the gene products were of the correct size.

When a gene product was of the correct size they were PCR purified by Qiagen's PCR purification kit and transformed into One Shot competent E. coli cells via a TOPO Cloning kit from Invitrogen. The transformed clones were then added to LB media and grown overnight. Clones were growth had occurred were then subjected to Qiagen's mini prep kit to harvest the DNA. Protocols for both of these procedures can be found in the appendices. The mini prep clones are then subjected to PCR with M13 primers and run on 1.5% agarose gels. The above mentioned M13 primer sequences and PCR reaction conditions are listed in the appendices. Gel electrophoresis images are provided in the appendices in one of my PowerPoint presentations to illustrate which clones were selected to be sequenced.

Sequencing of the positive mini prep clones was completed at Davis Sequencing which is located at the University of California at Davis. A total of 31 clones were sent off for sequencing and six yielded positive results. The six genes were determined by plugging the sequences of my clones into BLAST and searching for homologous genes. The other clones were determined to be artifacts of the cloning vector. The genes that were trapped were: human creatine kinase gene, human DORA reverse strand protein 1 (DREV1), human eukaryotic translation elongation factor 1 beta 2 (EEF1B2), human ribosomal protein L27, and human DNA clone RP11-290F20 on chromosome 20.

The gene-trapped clones that had yielded these sequencing results were expanded and RNA was collected from them for real-time PCR analysis. This was done to look at the gene expression of the trapped genes in the gene-trapped clones. These expression levels could then be compared to the parental cell line, MCF10A and a breast cancer cell line, MCF7. In the appendices I have included my real-time PCR protocol and the sequences for the primers and probes that were utilized. Also, graphical representations of the relative gene expression of my genes of interest are included.

KEY RESEARCH ACCOMPLISHMENTS

- A pooled library of gene-trapped of human mammary epithelial cells was established and frozen for future use.
- 92 out of 192 single cell assay gene-trapped clones were up- or down-regulated at least 2-fold in response to a 2.0 Gy radiation dose.
- Out of the 31 clones that were sequenced, six clones were found to be homologous to known genes through a BLAST search. These genes include: human creatine kinase gene, human DORA reverse strand protein 1 (DREV1), human eukaryotic translation

elongation factor 1 beta 2, human androgen receptor, human ribosomal protein L27, and human DNA clone RP11-290F20 on chromosome 20.

REPORTABLE OUTCOMES

- The development of a gene-trapped cell library of MCF10A cells was accomplished with the retrovirus pRET.
- I was invited to give a poster presentation at Colorado State University for the Cell and Molecular Biology Interdisciplinary Graduate Program Graduate Student and Post Doc Poster Competition on February 21, 2003. My poster abstract in found in the appendices.
- I was invited to give a 20 minute oral talk and a poster presentation at the Minority Trainee Research Forum sponsored by NIH-National Institute of Diabetes and Digestive Kidney Diseases, NIH-National Institute of Allergy and Infectious Diseases, NIH-Office of Research on Women's Health, and Merck and Company. It was held March 14-17, 2003 at the Westgate Hotel in San Diego, California. My abstract can be found in the appendices.
- On April 17, 2003 I gave a 20-minute oral presentation on my research to the faculty and students of the Department of Radiological and Environmental Health Sciences. The department has doctoral students give oral presentations every semester on how their research is progressing and any new findings. The PowerPoint slides from my presentation are given in the appendices.
- On November 21, 2002 I gave a 20-minute oral presentation on my research to the faculty and students of the Department of Radiological and Environmental Health Sciences.

Appendices

Primer sequences used for 3'RACE & Sequencing:
AD Poly (T): 5'-
CGTAGCTCTAGACTCCGTGTCCAACTTTTTTTTTTTTTTTTTTT-3'
AD (T): 5'-CGTAGCTCTAGACTCCGTGTCCAACT-3'
NEO1.5: 5'-GCGAATGGGCTGACCGCTTCCTCGTGC-3'
AD: 5'-CGTAGCTCTAGACTCCGTGTCCAAC-3'
NEO2.0: 5'-TACGGTATCGCCGCTCCCGATTCGCAG-3'
AD PLUS: 5'-CGTAGCTCTAGACTCCGTGTCCAACTTTT-3'
NEO SEQ: 5'-TGACGAGTTCTTCTGAGGGGATCC-3'
M13 Forward: 5'-GTAAAACGACGGCCAG-3'
M13 Reverse: 5'-CAGGAAACAGCTATGAC-3'

Primer and Probe sequences for real-time PCR:
Androgen F1: 5'-CCCTGGCGGCATGGT-3'
Androgen F2: 5'-ACCCTGGCGGCATGGT-3'
Androgen F3: 5'-TACCCTGGCGGCATGGT-3'
Androgen R1: 5'-CCCATTTCGCTTTTGACACA-3'
Androgen R2: 5'-CCCATTTCGCTTTTGACACAA-3'
Androgen R3: 5'-GCCCATTTCGCTTTTGACA-3'
DORA F1: 5'-GAGGCAGGGTCATCCTTGC-3'
DORA F2: 5'-GAGCCAACTAGAGGCAGGGTC-3'
DORA F3: 5'-GCCAACTAGAGGCAGGGTCA-3'
DORA R1: 5'-CCCACTTGCCACCTACGTTT-3'
DORA R2: 5'-TCCCACTTGCCACCTACGTT-3'
DORA R3: 5'-CTCCCACTTGCCACCTACGT-3'
ANDROGEN PROBE: 6FAM-AGCAGAGTGCCCTATCCCAGTCCA-TAMRA
DORA PROBE: 6FAM-CTTGTCCTCCCCTTTCATCCCTATGTGG-TAMRA
CK F1: 5'-TGCTACCATGGGCACCAGT-3'
CK F2: 5'-TTGCTACCATGGGCACCACT-3'
CK F3: 5'-TTGCTACCATGGGCACCAG-3'
CK R1: 5'-GCACACACTTTCTGCCGGT-3'
CK R2: 5'-GCACACACTTTCTGCCGGTT-3'
CK R3: 5'-GGCACTCGGCCATGCA-3'
L27 F1: 5'-GCCCCTACAGCCATGCTCT-3'
L27 F2: 5'-ATCGCCCTACAGCCATG-3'
L27 F3: 5'-TCAGATCGCCCCTACAGCC-3'
L27 R1: 5'-CATGGCAGCTGTCACTTTGC-3'
L27 R2: 5'-CCCATGGCAGCTGTCACTT-3'
L27 R3: 5'-TCTTGGCGATCTTCTTCTTGC-3'
EEF1B2 F1: 5'-CACAATTTGCGCGCTCTCT-3'
EEF1B2 F2: 5'-CCACAATTTGCGCGCTCT-3'
EEF1B2 F3: 5'-CCACAATTTGCGCGCTC-3'
EEF1B2 R1: 5'-ACCCATGGTGTCGGCTGTA-3'
EEF1B2 R2: 5'-ACCCATGGTGTCGGCTGT-3'

EEF1B2 R3: 5'-AACCCATGGTGTCGGCTGTA-3'
CK PROBE: 6FAM-TCCTGACCACCGGGTACCTGCTG-TAMRA
L27 PROBE: 6FAM-TGGCTGGAATTGACCGCTACCCC-TAMRA
EEF1B2 PROBE: 6FAM-TCTGCTGCTCCCCAGCTCTCGC-TAMRA

For the protocols used from Qiagen, Invitrogen and BD Biosciences, please visit the following websites:
http://www.invitrogen.com/content/sfs/manuals/topotaseq_man.pdf (TOPO TA Cloning)
http://www.invitrogen.com/content/sfs/manuals/18373019.pdf (3'RACE)
http://www1.qiagen.com/literature/handbooks/PDF/DNACleanupAndConcentration/QQ_Spin/1021422_HBQQSpin_072002WW.pdf (PCR purification)
http://www1.qiagen.com/literature/handbooks/PDF/RNAStabilizationAndPurification/FromAnimalAndPlantTissuesBacteriaYeastAndFungi/RNY_Mini/1016272HBRNY_062001WW.pdf (RNeasy Mini Kit)
http://www1.qiagen.com/literature/handbooks/PDF/PlasmidDNAPurification/PLS_QP_Miniprep/1023790_HBQP_Miniprep_0303.pdf (Miniprep kit)
http://www.clontech.com/techinfo/manuals/PDF/PT1580-1.pdf (Advantage-GC cDNA Polymerase kit)

General Protocol for the Sandwich ELISA method:

1. Before the assay, both antibody preparations should be purified and one must be labeled.
2. For most applications, a polyvinylchloride (PVC) microtiter plate is best; however, consult manufacturer guidelines to determine the most appropriate type of plate for protein binding.
3. Bind the unlabeled antibody to the bottom of each well by adding approximately 50 m L of antibody solution to each well (20 m g/mL in PBS). PVC will bind approximately 100 ng/well (300 ng/cm2). The amount of antibody used will depend on the individual assay, but if maximal binding is required, use at least 1 m g/well. this is well above the capacity of the well, but the binding will occur more rapidly, and the binding solution can be saved and used again.
4. Incubate the plate overnight at 4° C to allow complete binding.
5. Wash the wells twice with PBS. A 500 mL squirt bottle is convenient. The antibody solution washes can be removed by flicking the plate over a suitable container.
6. The remaining sites for protein binding on the microtiter plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2hrs to overnight in a humid atmosphere at room temperature. (Note: Sodium azide is an inhibitor or horseradish peroxidase. Do not include sodium azide in buffers or wash solutions, if an HRP-labeled antibody will be used for detection.)
7. Wash wells twice with PBS.
8. Add 50 m L of the antigen solution to the wells (the antigen solution should be titrated). All dilutions should be done in the blocking buffer (3% BSA/PBS with

0.02% sodium azide). Incubate for at least 2 hrs at room temperature in a humid atmosphere.

9. Wash the plate four times with PBS.
10. Add the labeled second antibody. The amount to be added can be determined in preliminary experiments. For accurate quantitation, the second antibody should be used in excess. All dilutions should be done in the blocking buffer.
11. Incubate for 2 hrs or more at room temperature in a humid atmosphere.
12. Wash with several changes of PBS.
13. Add substrate as indicated by manufacturer. After suggested incubation time has elapsed, optical densities at target wavelengths can be measured on an ELISA reader.

PCR conditions:

First strand cDNA synthesis:

94°C 3 minutes; 94°C 40 seconds, 72°C 4 minutes, 8 cycles; 94°C 40 seconds, 66°C 2 minutes, 72°C 2 minutes, 32 cycles; 72°C 4 minutes, 4°C overnight.

Second strand cDNA synthesis:

94°C 1 minute, 94°C 40 seconds, 72°C 4 minutes, 8 cycles; 94°C 40 seconds, 66°C 2 minutes, 72°C 2 minutes, 32 cycles; 72°C 4 minutes, 4°C overnight.

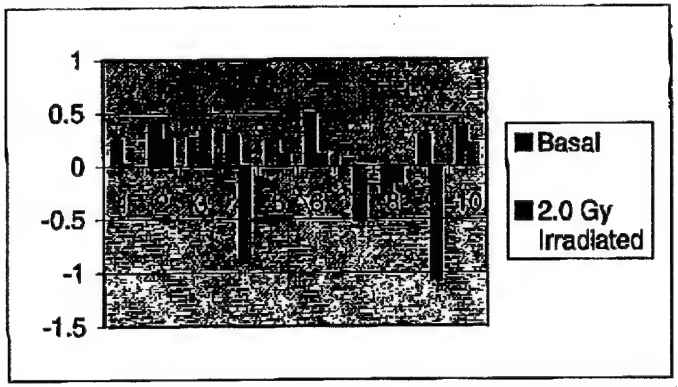
Mini prep PCR:

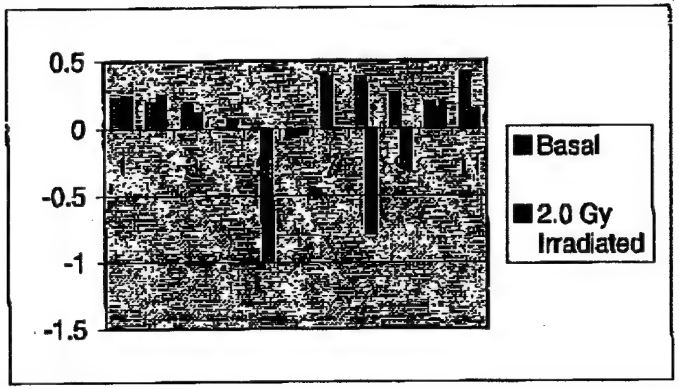
94°C 2 minutes; 94°C 1 minute, 55°C 1 minute, 72°C 1 minute, 25 cycles; 72°C 7 minutes.

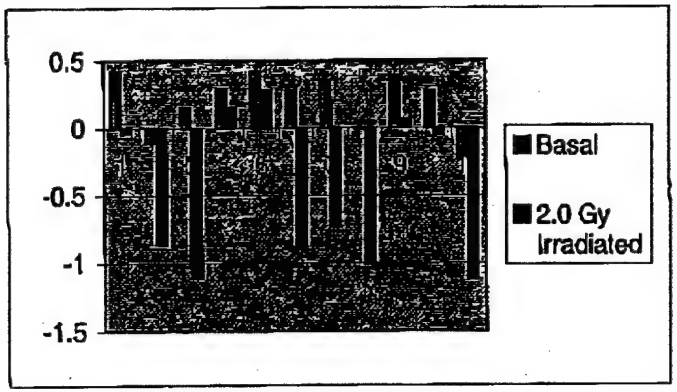
Real-time PCR:

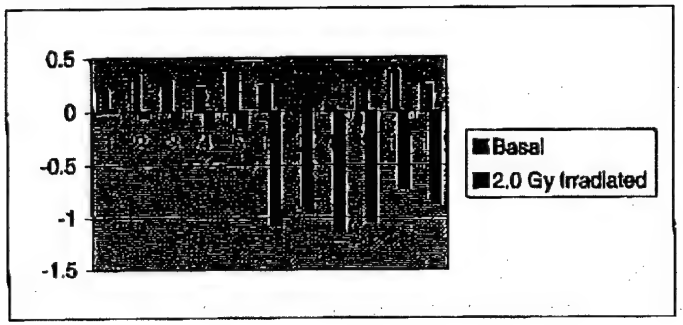
50°C 2 minutes; 95°C 10 minutes; 95°C 15 seconds, 60°C 1 minute, 60 cycles.

0.247	0.023
0.382	0.251
0.268	0.336
0.276	-0.851
0.221	0.105
0.494	0.104
0.057	-0.515
-0.245	-0.154
0.285	-1.059
0.353	0.205
0.228	0.238
0.194	0.249
0.18	0.115
0.012	0.068
0.004	-0.975
-0.065	-0.044
0.38	-0.009
0.382	-0.787
0.258	-0.297
0.197	0.15
0.414	0.137
0.423	-0.036
-0.105	-0.859
0.152	-1.104
0.284	0.153
0.418	0.28
0.273	-0.85
0.346	-0.729
-0.011	-1.012
0.322	0.063
0.276	-0.047
-0.216	-1.117
0.194	0.021
0.337	-0.049
0.279	-0.031
0.225	-0.156
0.349	-0.139
0.238	-1.067
-0.009	-0.894
0.047	-1.132
0.195	-1.037
0.374	-0.723
0.241	-0.869
0.343	0.166
0.13	-0.879
0.306	-0.824





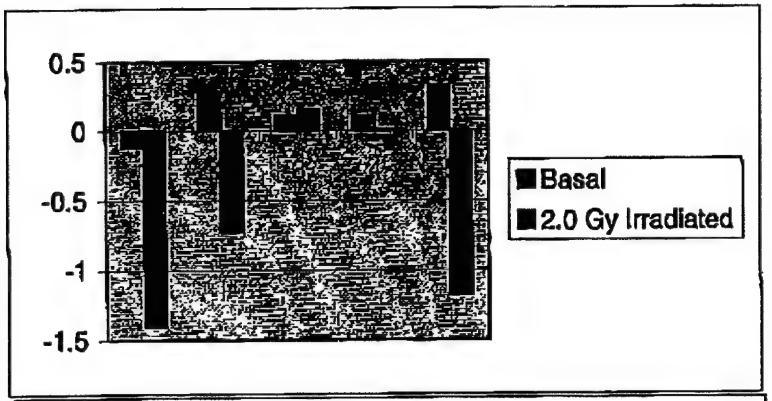


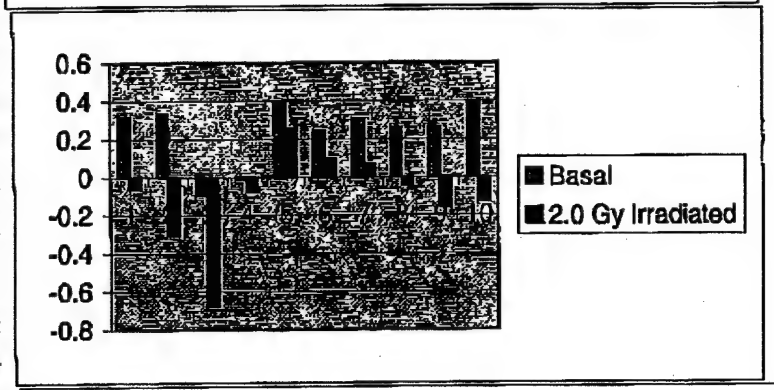


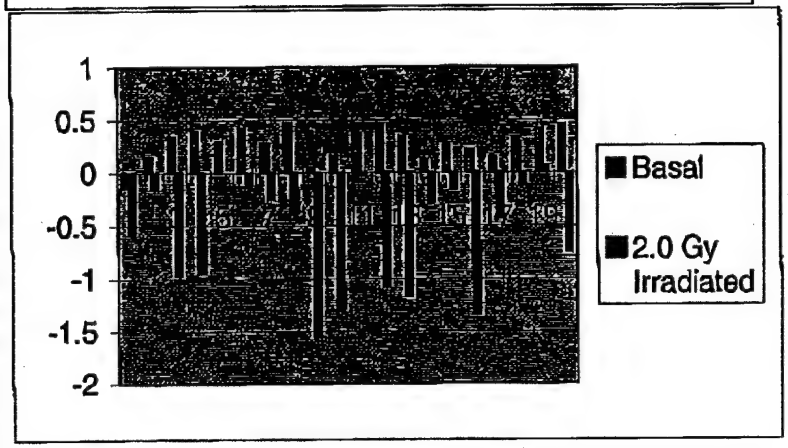
GFP negative sort population
GFP readings with microplate reader
both before & after 2.0 Gy irradiation.

0.294	-0.999
0.221	-1.079
0.126	0.215
0.38	0.28
0.479	-0.635
0.261	0.237
0.411	0.638
0.237	-0.87
0.355	-0.71
-0.115	-0.11
0.263	0.169
0.463	-0.855
0.267	0.174
0.308	0.254
0.187	0.044
0.411	-0.353
0.208	-0.046
0.001	0.091
0.289	-0.969
0.459	-0.86
0.518	-0.698
0.214	0.086
0.457	0.12
0.316	-0.155
0.525	-0.838
-0.019	-0.092
0.022	-0.002
0.007	-0.977
0.139	-0.211
0.044	-0.158
0.229	-0.251
0.106	-0.27
0.021	-0.435
0.297	-1.245

-0.117	-1.403
0.253	-0.726
0.109	0.154
0.1	0.007
0.326	-1.178
0.317	-0.061
0.334	-0.305
-0.094	-0.676
-0.011	-0.076
0.398	0.255
0.242	0.093
0.306	0.067
0.255	-0.043
0.26	-0.159
0.395	-0.124
-0.136	-0.559
0.15	-0.151
0.345	-0.97
0.391	-0.944
0.291	0.001
0.419	-0.091
0.278	-0.246
0.467	-0.374
0.031	-1.554
0.153	-1.286
0.367	-0.026
0.457	-1.07
0.334	-1.169
0.119	-0.283
0.252	-0.156
0.216	-1.341
0.143	-0.429
0.329	-0.095
0.404	0.06
0.451	-0.744
0.362	-0.022
0.377	-0.808



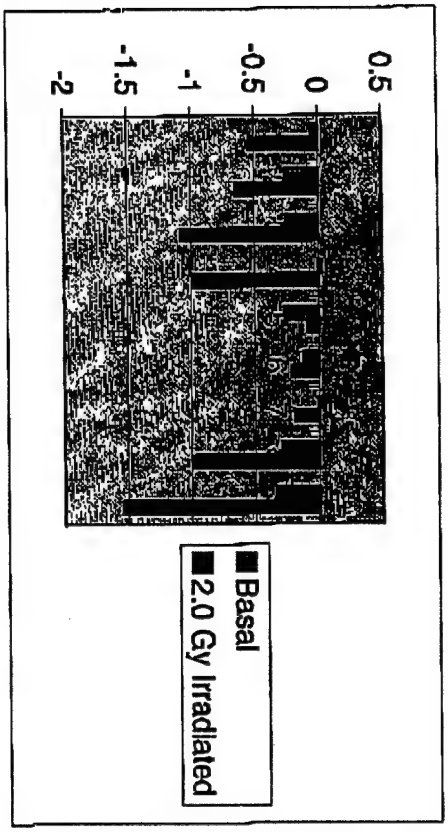




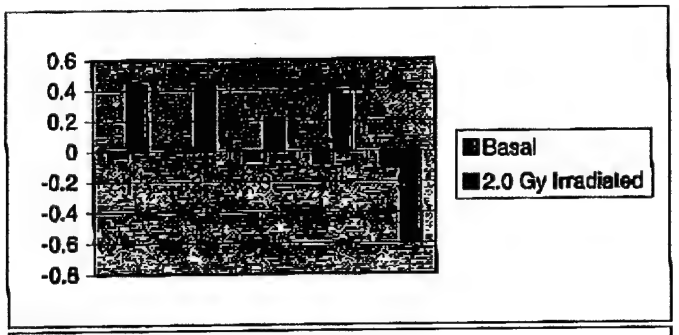
GFP positive sort population
GFP readings with microplate reader
both before & after 2.0 Gy irradiation.

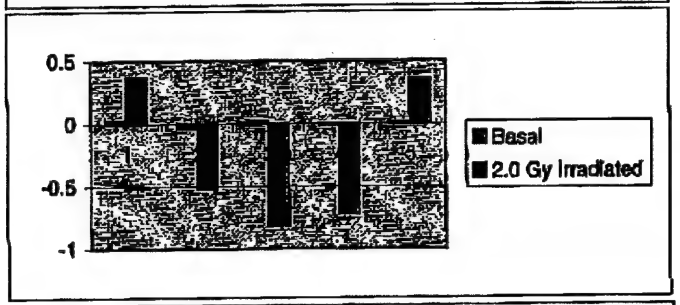
0.222	-0.154	
0.164	0	
-0.064	-1.559	
0.293	-1.272	
0.496	0.34	
0.503	-0.04	
0.441	-0.506	
0.266	-1.17	
0.28	-1.089	
0.234	-0.238	
0.044	-0.303	
0.102	-0.252	
0.304	-0.556	
0.552	0.019	
0.513	-1.182	
0.368	-1.17	
0.357	-0.297	
0.511	-0.31	
-0.092	-0.687	
0.237	-0.383	
0.556	0.124	
0.51	-0.01	
0.559	0.038	
0.359	-0.182	
0.534	-1.096	
0.224	-1.274	
0.239	-0.392	
0.301	-0.497	
0.427	-0.028	
0.543	0.032	
0.362	-0.147	
0.299	-0.084	
0.204	-1.245	
0.232	-0.212	
-0.052	-0.539	
-0.258	-0.647	
-0.256	-1.081	

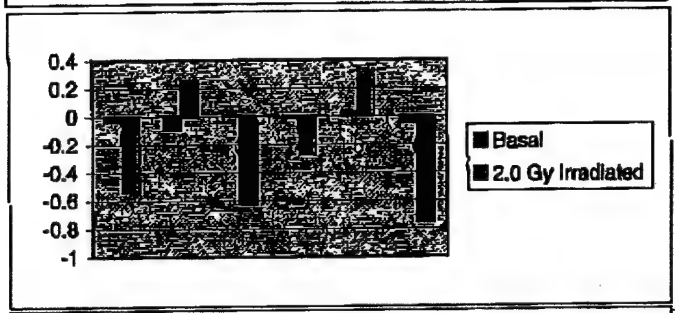
0.025	-0.984
-0.268	-0.086
-0.123	-0.214
-0.01	-0.194
-0.293	-0.995
-0.337	-1.541

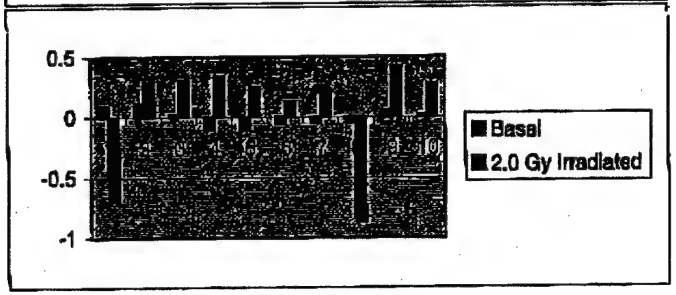


-0.036	0.433
-0.004	0.414
-0.064	0.218
-0.08	0.362
-0.109	-0.584
0.024	0.372
-0.031	-0.518
0.024	-0.814
-0.01	-0.721
0.01	0.357
-0.037	-0.532
-0.091	0.256
0.007	-0.627
-0.015	-0.273
0.03	0.289
-0.05	-0.755
0.089	-0.689
0.111	0.291
0.022	0.301
-0.102	0.34
-0.101	0.25
-0.052	0.126
-0.046	0.186
-0.05	-0.87
0.052	0.422
0.019	0.279
0.11	-0.61
0.031	0.347
-0.075	0.138
-0.05	-0.785
0.11	-0.732
-0.04	-0.929







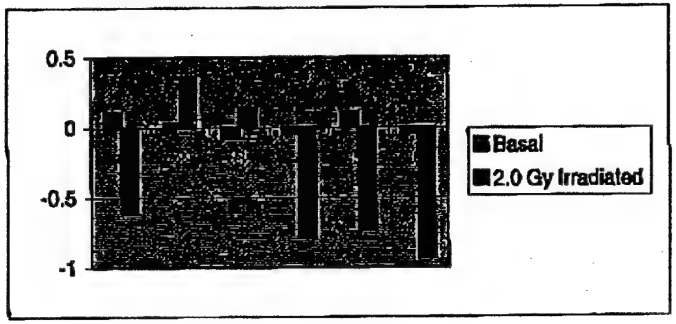


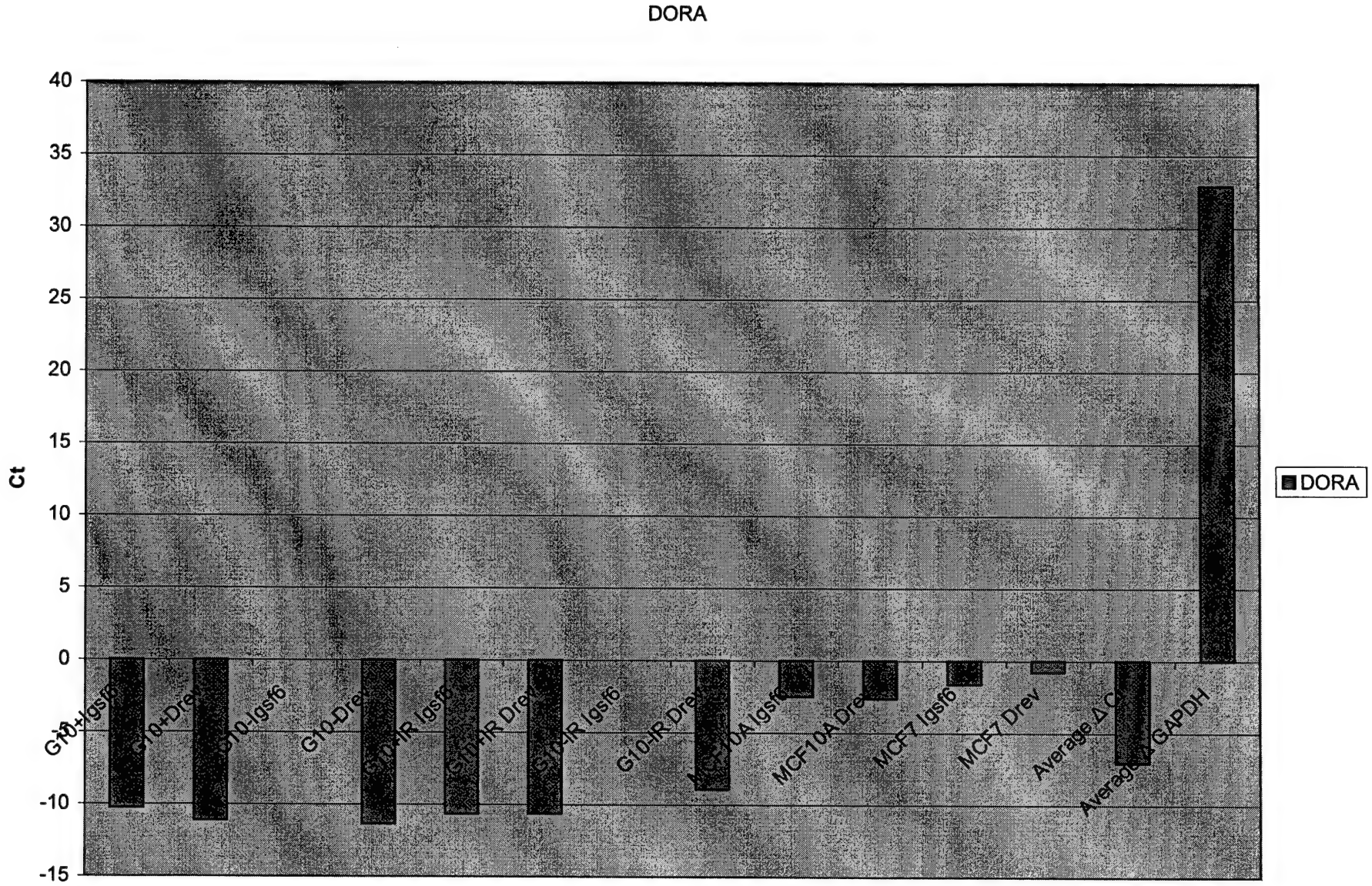
GFP positive Lanes A1 through A16
GFP negative Lanes A17 through A32

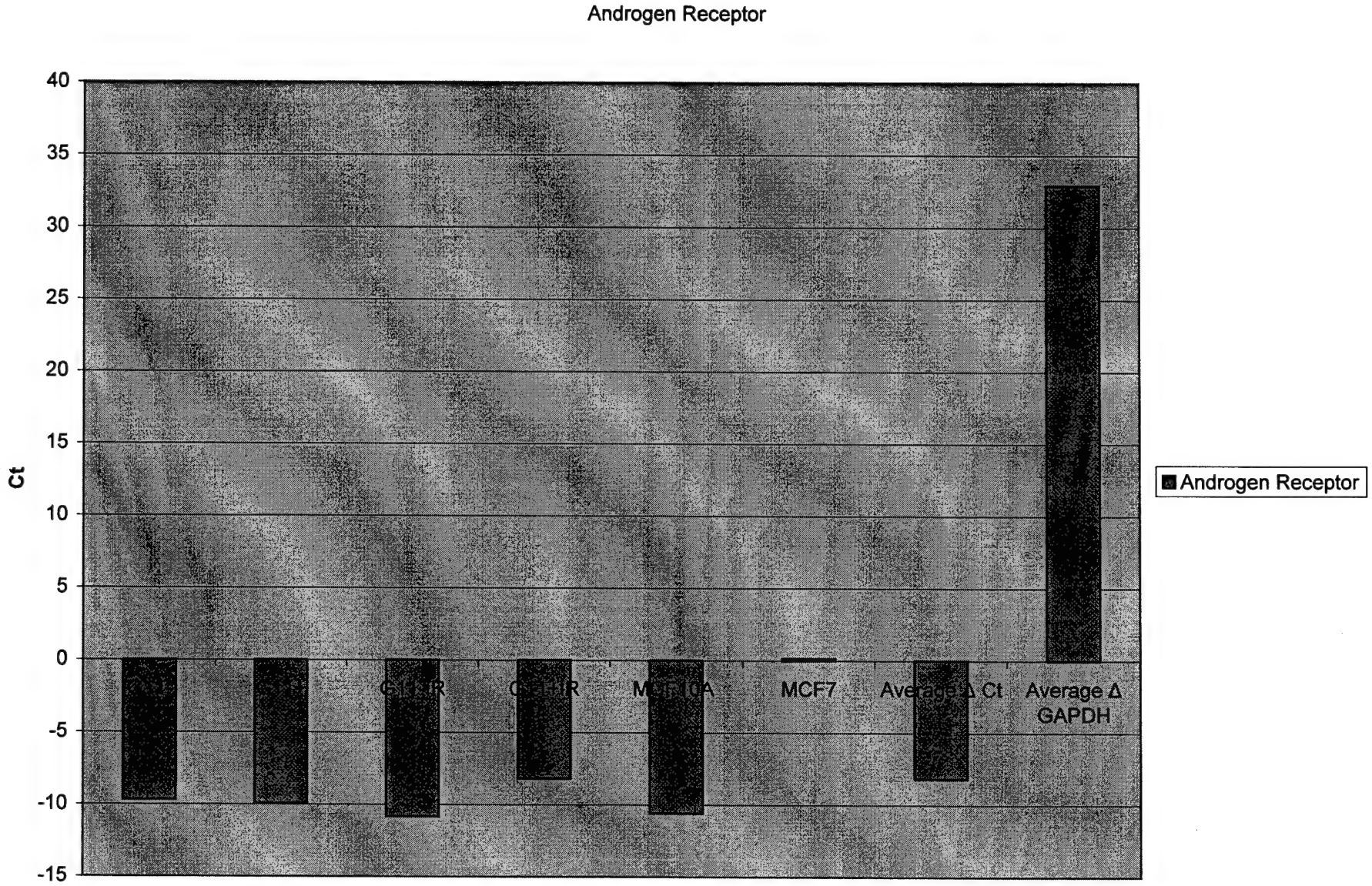
GFP readings from microplate reader
for both before & after 2.0 Gy irradiation.

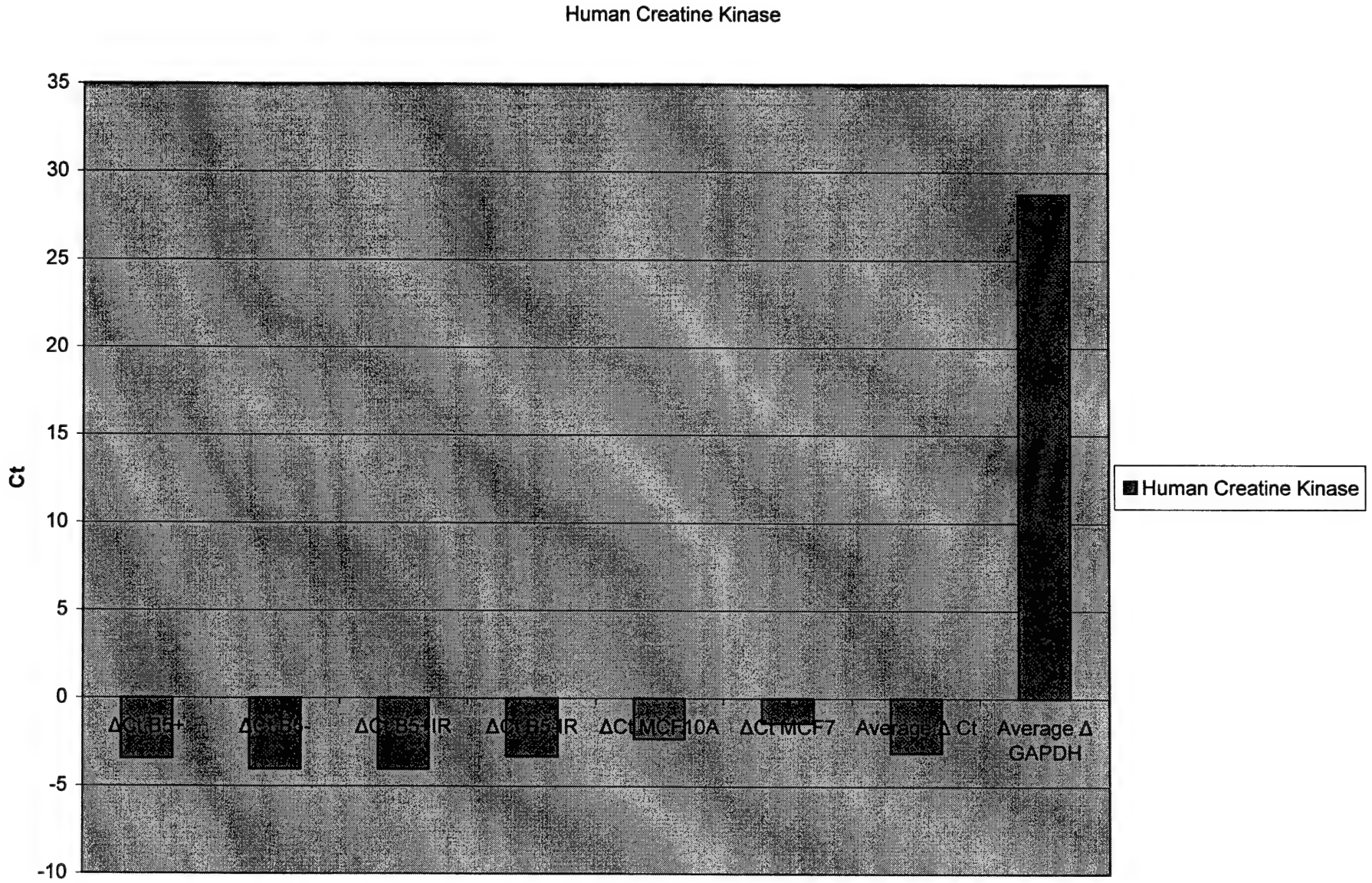
Each sample listed to the right is one individual clone of the 192 clones sampled.

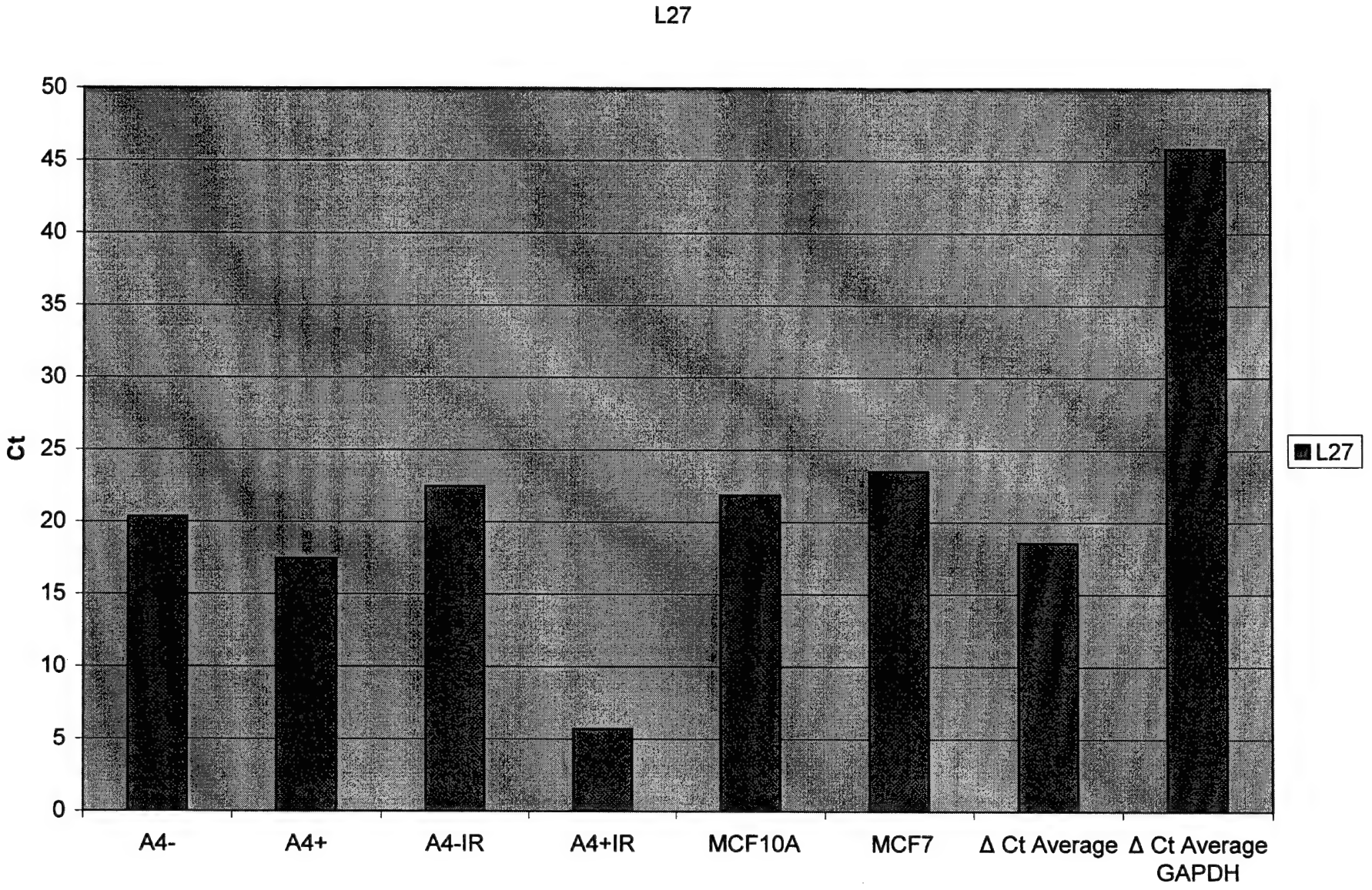
Those clones up- or down-regulated at least 2-fold were expanded for further analysis.




DORA
40
35
30
25
20
15
10
5
0
-10
-15
Ct
DORA
G10+Igsf6
G10+Drev
G10-Igsf6
G10-Drev
G10-IR Igsf6
G10-IR Drev
G10-IR Igsf6
G10-IR Drev
MCF10A Igsf6
MCF10A Drev
MCF7 Igsf6
MCF7 Drev
Average ΔC
Average ΔΔ GAPDH


Androgen Receptor
40
35
30
25
20
15
10
5
0
-5
-10
-15
Ct
MCF7
Average Δ Ct
Average Δ GAPDH
Androgen Receptor


Human Creatine Kinase
35
30
25
20
15
10
5
0
-5
-10
Ct
ΔCt B5+
ΔCt B5-
ΔCt B5+IR
ΔCt B5-IR
ΔCt MCF10A
ΔCt MCF7
Average Δ Ct
Average Δ GAPDH
Human Creatine Kinase


L27
50
45
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25
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15
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5
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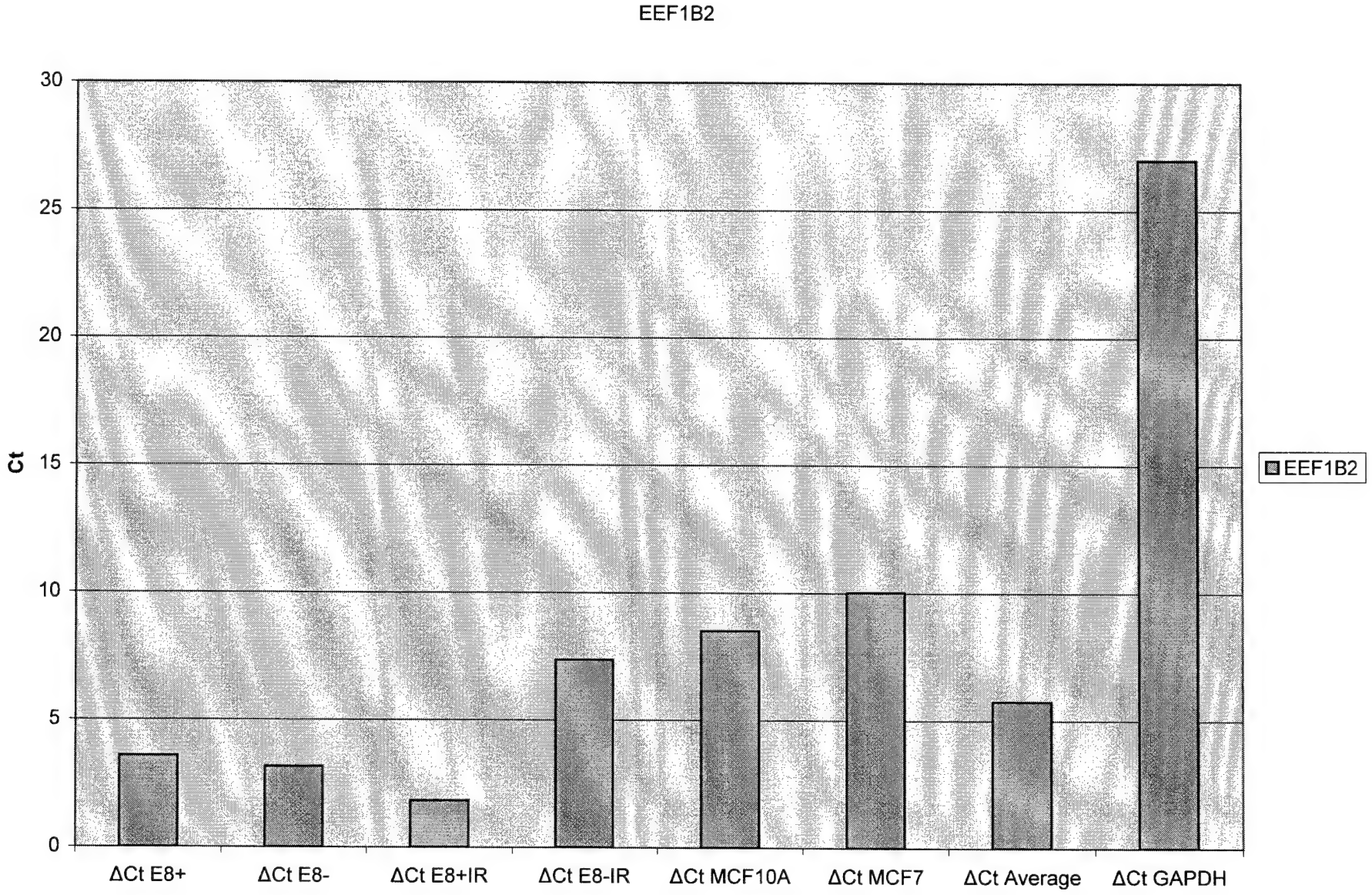
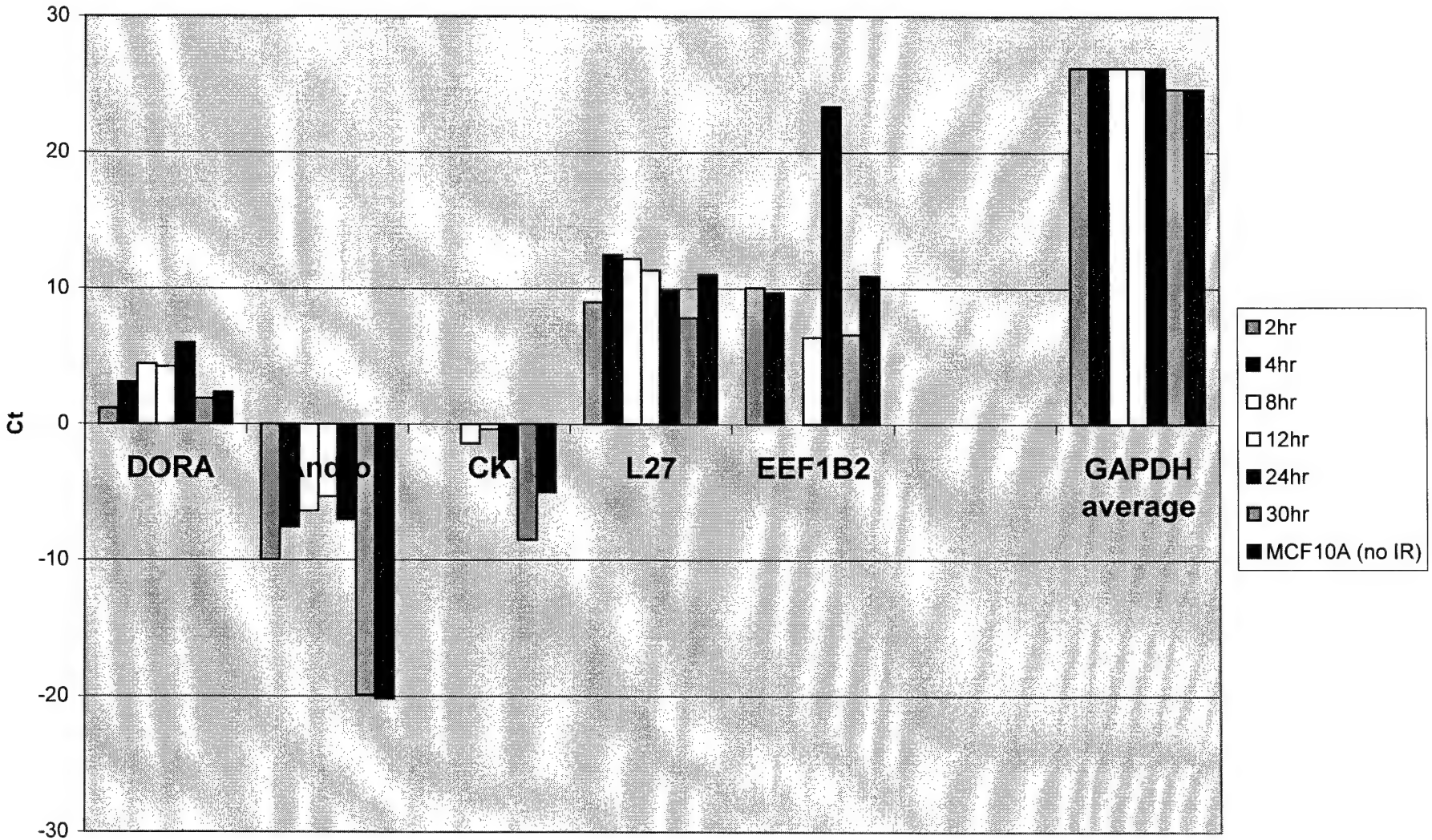
Ct
A4-
A4+
A4-IR
A4+IR
MCF10A
MCF7
Δ Ct Average
Δ Ct Average GAPDH
L27


EEF1B2
30
25
20
15
10
5
0
Ct
ΔCt E8+
ΔCt E8-
ΔCt E8+IR
ΔCt E8-IR
ΔCt MCF10A
ΔCt MCF7
ΔCt Average
ΔCt GAPDH
EEF1B2


Gene Expression after 2.0Gy irradiation dose
30
20
10
0
-10
-20
-30
Ct
DORA
CK
L27
EEF1B2
GAPDH
average
2hr
4hr
8hr
12hr
24hr
30hr
MCF10A (no IR)

Cell and Molecular Biology Interdisciplinary Graduate Program

Graduate Student & Post Doc Poster Competition Abstracts

Spring 2003

Colorado State University

IDENTIFICATION OF NOVEL GENES AFFECTED BY GAMMA IRRADIATION USING A GENE-TRAPPED LIBRARY OF HUMAN MAMMARY EPITHELIAL CELLS

Jennifer Malone and Robert Ullrich
Department of Environmental and Radiological Health Sciences, Colorado State University
malones41500@hotmail.com, 491-7497 office, 491-5771 lab, classification: graduate student

Objective/Hypothesis: In this study, we plan to establish an assay to identify novel genes that are affected by gamma irradiation and to characterize their function and role in early breast carcinogenesis. We hypothesize that the mutation of these genes or their abnormal expression in response to gamma irradiation is one of the causes of breast carcinogenesis.

Specific Aims: The specific aims of this study are:

1. To establish a high throughput assay for detection of variation in gene expression in human mammary epithelial cells using gene-trapped MCF10A clones; 2. To determine the effect of gamma irradiation on the expression of reporter protein green fluorescent protein (GFP); 3. To characterize the effect of gamma irradiation on transformation of human mammary epithelial cells; 4. To identify the trapped genes affected by gamma irradiation in breast epithelial cells.

Methods: We plan to establish an assay that will allow us to screen for breast cells that contain a single gene mutation using a technique called gene trapping. We will be able to detect changes in the expression of a specific gene upon treatment with different doses of radiation. These radiation-responsive genes will be identified through the rapid amplification of cDNA ends (RACE) procedure and sequenced. Cells that are affected by radiation will be isolated and further analyzed to see if the changes can lead to the malignant transformation of the normal breast epithelial cell into a neoplastic cell.

Results: The MCF10A gene-trapped library has been established. Basal GFP levels have been measured from the replica plates of single cell clones in 96-well plates. Gamma irradiation of the single cell clones at 2.0 Gy has been performed. Clones that were either up or down-regulated in response to the radiation treatment will be expanded for further analysis.

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Conclusions: This assay may prove to be a powerful tool in the identification of novel genes that are affected by gamma irradiation in the early stages of breast cancer progression. This study will provide new information on the effects of radiation-responsive genes that can lead to breast cancer as well as to identify new markers for early detection of breast cancer. This study will focus on the identification of novel genes that are potential targets of gamma irradiation. It will provide essential information on the immediate and long-term effects of gamma irradiation of breast cells that may be the key to further understanding of the mechanism of radiation-induced breast cancer.

Program & Schedule

Minority Trainee Research Forum

Invitational Scientific Presentations by Minority Biomedical Trainees from Academic, Industry, and Government Labs

March 14–17, 2003
The Westgate Hotel
San Diego, CA

Invitees:
Postdoctoral Fellows
Graduate Students
M.D./Ph.D. Students
Medical Students
Undergraduate Students
High School Students

Sponsored by:
- NIH – National Institute of Diabetes and Digestive and Kidney Diseases
- NIH – National Institute of Allergy and Infectious Diseases
- NIH – Office of Research on Women's Health
- Merck & Company

March 17, 2003

Registration

7:00 a.m. – 5:00 p.m.

Foyer

Breakfast Seminar I
High School; College; PostBac Trainees

7:00 a.m. – 8:25 a.m.
Temple University

Regency
Preparing for Medical/Graduate School

Breakfast Seminar II
M.D./Ph.D.; M.D.; Ph.D.; PostDoc Trainees

7:00 a.m. – 8:25 a.m.
UNCF/MERK

Le Fontainebleau Restaurant
Science Education Initiatives

Oral Presentations

8:30 a.m. – 11:30 a.m.

Versailles Ballroom

8:30 **Jill White (IL)**
Ph.D. Trainee
Northwestern University

"Differential Induction of Gilal-mediated Inflammation by Oligomeric Versus Fibrillar Aβ1-42"

8:45 **Wayne Lilyestrom (CO)**
Ph.D. Trainee
University of Colorado Health Sciences Ctr.

"Biochemical and Structural Characterization of SV40 Large-T Antigen"

9:00 **Crystal Johnson (AL)**
Ph.D. Trainee
University of Alabama-Birmingham

"Genetic Relatedness of Levofloxacin-Resistant Streptococcus Pneumoniae Isolates from North America"

9:15 **Fabrico Rojas (CA)**
Ph.D. Trainee
California State University, Northridge

"Characterization of the Carbofuran Hydrolase Promoter in Achromobacter"

9:30 **Jennifer Malone (CO)**
Ph.D. Trainee
Colorado State University

"Identification of Novel Genes affected by Gamma Irradiation using a Gene-trapped Library of Human Mammary Epithelial Cells"

9:45 **Tyisha Williams (MD)**
Ph.D. Trainee
Howard University

"Mutation Analysis of Glutathione Reductase a Candidate Gene for Skin Color"

10:00 **Angelia Lockett (IN)**
Ph.D. Trainee
Indiana University & Purdue University

"Is LPS Induction of NFkB Activity Indirect and Due to TNFa?"

10:15 **Kevin Hadley (CA)**
PostDoc
Children's Hospital of Oakland Research Institute

"Effect of β-thalassemia, Sickle-Cell Disease and Dietary Iron on Iron Storage in Transgenic Mice"

10:30 **Lee Wilson (Canada)**
PostDoc
University of Saskatchewan

"Interaction of Inhalation Anesthetics with Supramolecular Biomimetic Structures"

10:45 **Melissa Gonzales (CO)**
PostDoc
University of Colorado Health Science Ctr.

"Nordihydroguaiaretic Acid Inhibits UVB-induced AP-1 Activation in Human Keratinocytes"

11:00 **Jesus Salvador (MD)**
PostDoc
National Cancer Institute

"A Role for the p53-effector Gene Gadd45a as an Autoimmune Disease Suppressor Gene"

11:15 **Laundette Jones (MD)**
PostDoc
Lombardi Cancer Center,
Georgetown University

"Cellular and Molecular Alterations Associated with Early and Late Stages of Mammary Tumor Progression in Conditional Brca1 Exon 11 Deficient Mice"

IDENTIFICATION OF NOVEL GENES AFFECTED BY GAMMA IRRADIATION USING A GENE-TRAPPED LIBRARY OF HUMAN MAMMARY EPITHELIAL CELLS

Jennifer Malone and Robert Ullrich
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Using a gene-trapped library of MCF10A cells to identify novel genes affected by gamma IR

Jennifer Malone
Colorado State University
November 21, 2002

Overview of presentation

- Introduction
- Experimental Design
- Future Directions
- Summary
- Acknowledgements

Introduction

Breast Cancer

- Most common malignancy in women in the US and second leading cause of cancer deaths in women (193,706 new cases in 2001 among women in the United States and about 40,600 deaths)
- Both genetic and environmental components associated with disease.
- Up to 10% of breast cancer cases are due to genetic mutations in such genes as BRCA1, BRCA2, and ATM.

Gamma Irradiation and Breast Cancer

- Induced with high frequency by radiation.
- Ionizing radiation is one of the main treatments used to manage & kill cancer cells.
- Breast cancer risk seen in women exposed to total doses of more than 1 Gy.
- Exposure prior to age 19 leads to increased incidence of breast cancer, but exposures after menopause doesn't effect risk.
- Radiation treatments for breast cancer linked to increased risks of secondary breast cancers among women exposed prior to the age of 45

What is Gene Trapping?

- A method of random insertional mutagenesis that uses a fragment of DNA coding for a reporter or selectable marker gene as a mutagen to specially disrupt gene function by producing intragenic integration events.
- Gene trapping allows the simultaneous identification, sequencing, in vivo expression analysis, and phenotyping of the genes of interest.

Gene Trapping

- The sequence of the 'trapped' gene can be identified using techniques that are based on the polymerase chain reaction (PCR), and this can lead to the isolation of novel genes regardless of their level of expression in vivo.

Methods for introduction of vectors into cells

- Electroporation: used for DNA transformation. Relatively inefficient.
- Retroviral vectors: high efficiency of gene transfer and integrate as a single copy.

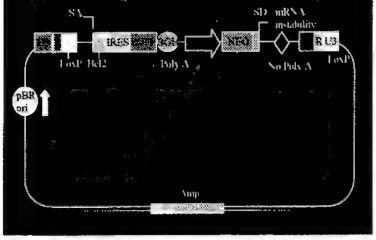
Retroviral gene trapping vectors

- The vectors used in this approach typically include a splice acceptor site upstream of a promoterless reporter gene and the selectable *neo* gene driven by an autonomous promoter.
- A retroviral vector is introduced into packaging cell lines by transfection. Viral supe is then used to infect MCF10A cells.
- Reporter gene activity reflects the activity of the endogenous gene into which the vector integrated.

Removable Exon Trap (RET)

- A poly-A trap retrovirus vector constructed by Ishida and Leder uses a combination of a very strong splice acceptor, an effective polyadenylation signal and a promoterless green fluorescent protein cDNA that allows the expression pattern of the trapped gene to be monitored in living cells.
- The integrated provirus can be removed from the genome of infected cells by excision using Cre homologous recombination due to its loxP sites.

Map of pRET1023



The Process of Vector Integration

- The vector is randomly integrated into the genome in regions where functional genes are found.
- The random insertion of a retroviral vector is designed to signal its presence via the activation of the reporter gene, which both mimics the expression of the endogenous gene and potentially mutates the locus.
- Endogenous gene locus is usually (but not always) inactivated by vector integration, leading to a loss of function.

Experimental Design

Cell Sorting of Infected pool of GFP clones

- Cells sorted through flow cytometer in Dr. Fox's laboratory.
- MCF10A clones were sorted by GFP expression into positive and negative pools.
- The positive GFP expression pool was then further sorted into high, medium, and low levels of GFP expression.

Replica Plating

- Pooled clones of gene-trapped MCF10A clones plated by limiting dilution
- (1 cell/well) in 96-well plates.
- Expand in presence of G418
- Once confluent, will replica plate to 6-96 well plates per each GFP- and GFP + sort clonal cell population.
- The 6 plates will be for: basal GFP detection, master, store at – 80 degrees, 2 0 Gy , 0 5 Gy, and control.

Positive GFP expression in MCF10A-1023 I_6 clone 109 from Replica Plating

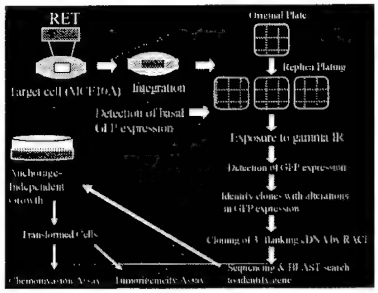
Detection of basal GFP expression using microplate reader

- BioRad microplate reader will be used to quantitate GFP expression.
- 96-well plates scanned at 450 nm to detect GFP by the use of TMB substrate.
- Absorbance quantitated using Microplate manager software.

Future Directions

Exposure to gamma irradiation

- MCF10A clones will be subjected to varying low doses (0-2 Gy) of gamma irradiation by a sealed ^{137}Cs source.
- Gamma irradiation is a known mutagenic agent.
- GFP expression quantified with microplate reader immediately following radiation exposure.



3' RACE Assay

- Rapid Amplification of cDNA ends amplifies nucleic acids sequences from a mRNA template between a defined internal site & either the 3' or 5' end of the mRNA.
- Uses natural polyA tail found in mRNA as a generic priming site for PCR.
- mRNAs converted to cDNA using reverse transcriptase and an oligo-dT adapter primer.
- cDNA amplified by PCR using a gene-specific primer that anneals to a region of known exon sequences and an adapter primer that targets the polyA tail region.

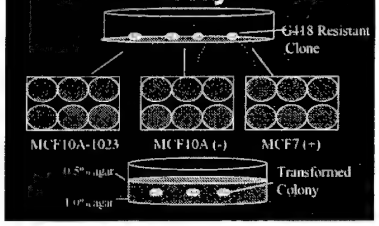
3' RACE continued

- Permits capture of unknown 3' mRNA sequences that lie between the exon and polyA tail.
- PCR products cloned into sequencing vector using Zero Blunt TOPO Cloning kit from Invitrogen.
- Nucleotide sequence of purified PCR fragments determined by sequencing with the M13 universal primers.
- Search BLASTX & BLASTN databases for homologous genes.

Soft Agar Assay for Anchorage Independence

- In 6-well plate: bottom layer contains 3 ml 1% agarose. Top layer contains 6 ml 0.5% agarose and 1.0 x 10^4 cells.
- Both layers contain appropriate media.
- Wait at least 2 weeks or more to detect colonies to detect transformation.

Summary of Soft Agar Assay



Chemoinvasion & Chemotaxis Assay

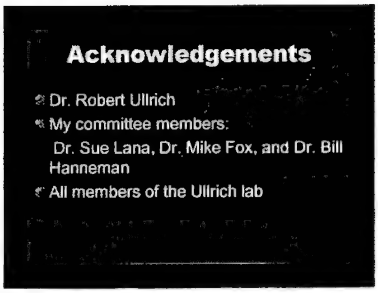
- Determine invasiveness using Boyden Chamber assay.
- Cells seeded in upper chamber & incubated 6 hrs.
- Conditioned media in lower chamber used as chemoattractant.
- Cells that crossed filter will be fixed & counted.
- Chemotaxis assay will serve as a control for invasion assay.

Tumorigenicity in nude mice

- To determine if anchorage-independent clones are fully malignant, MCF10A transformed cells will be injected subcutaneously into the subscapular area of 3-week old irradiated athymic female nude mice (BALB/c background).
- Palpable tumors monitored weekly.
- Sites of injection & palpable
- Tumors will be examined histologically.

Summary

- Focus on identification of novel genes that are potential targets of IR.
- Provide essential info on immediate & long-term effects of IR on breast cells which will lead to further understanding of the mechanism of radiation-induced breast carcinogenesis.
- This assay can be used to test other potential environmental risk factors.


Acknowledgements
Dr. Robert Ullrich
My committee members:
Dr. Sue Lana, Dr. Mike Fox, and Dr. Bill Hanneman
All members of the Ullrich lab

Gene Trapping: A Tool to Identify Novel Genes Affected by Gamma Irradiation in Breast Cancer

Jennifer Malone
Colorado State University
April 17, 2003

Hypothesis

- *Mutation of novel genes or their abnormal expression in response to a single dose of gamma radiation is one of the causes of early breast carcinogenesis.*

- **Specific aim 1.** To establish a high throughput assay for detection of variation in gene expression in human mammary epithelial cells using gene trapped MCF-10A clones.
- **Specific aim 2.** To determine the effect of gamma irradiation on expression of reporter protein GFP.
- **Specific aim 3.** To identify the "trapped" genes affected by gamma irradiation.
- **Specific aim 4.** To characterize the effect of gamma irradiation on transformation of human mammary epithelial cells.

Radiation-Induced DNA Damage

- The responses to ionizing radiation involve: the activation of signaling pathways, cell-cycle arrest, mutations, transformation, and cell death.
- Growth factors, cytokines, oncogenes, and genes involved in the cell cycle, apoptosis, and DNA repair are all known to be effected.
- Responses to radiation:
  - -generalized response to cellular injury (indirect)
  - -specific to radiation-induced damage (direct)

Known radiation-inducible genes

- IR works via DNA damage and ROS generation, which can induce the transcription of specific genes through the activation of p53, NF-kB and AP-1.
- Also known to be induced: GADD45A, CDKN1A (CIP1/WAF1), MDM2, ATF3, BAX
- Many known radiation-inducible genes have been found to be early response genes.

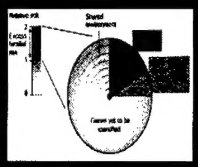
Breast Cancer& Radiation

- Breast cancer may be induced with relatively high frequency by radiation.
- Ionizing radiation is one of the main treatment modalities used in the management of cancer.
- Radiation is used in the treatment of breast cancer, leukemia, and Hodgkin's lymphoma to kill cancerous cells.
- Breast irradiation and drug administration have been prescribed to treat postpartum breast swelling.
- A radiation dose-related increase in the incidence of breast cancer has been seen in women.
- When a woman receives significant radiation prior to the age of 20, she becomes more likely to develop breast cancer.

Breast Cancer Info

- 2nd leading cause of cancer death (after lung cancer).
- There are both genetic and environmental components associated with breast cancer.
- Up to 10% of breast cancer in western countries is due to genetic predisposition.
- Breast cancer incidence increases strongly with age.

What genes might account for familial breast cancer?



Averaged across all ages, the risk of breast cancer to the sister, mother or daughter of a case is increased about twofold, as illustrated in the figure to the right. This excess familial risk provides an upper estimate (assuming all the risk is genetic) of the genetic effect that must be explained. Source: Pharoah, P., *Cancer genetics*. Nature, 2001

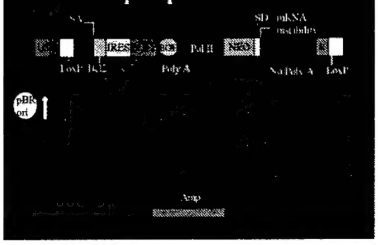
Gene Trapping

- Form of insertional mutagenesis.
- Disrupt gene function by intragenic integration.
- mRNA transcribed from a selectable marker gene lacking a poly-A signal in a gene-trap vector is stabilized only when the gene-trap vector captures a cellular poly A signal.
- Poly A trapping occurs independently of the expression of target genes, regardless of its expression.
- The sequence of the "trapped" gene can be identified using techniques that are based on the polymerase chain reaction (PCR), and this can lead to the isolation of novel genes regardless of their level of expression in vivo.

RET retrovirus

- Enhanced poly A-trap for stringent selection of viral integration.
- Strong splice acceptor and an efficient poly A signal included for the complete disruption of the trapped gene.
- Expression of trapped gene analyzed by GFP expression.
- Mutant phenotype verified by removing virus with Cre/loxP-mediated homologous recombination.

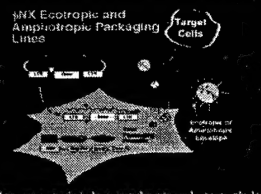
Map of pRET1023



MCF10A Gene Trapped Library Construction

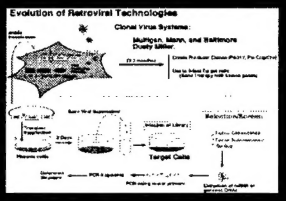
- pRET retrovirus transfected w/ Lipofectamine into Phoenix Amphotrophic mouse packaging cell line
- It is based on Moloney Murine Leukemia Virus (MMULV) and allows for delivery of genes to most dividing mammalian cell types.
- Amphotropic system (capable of delivering genes to dividing cells of most mammalian species, including human).
- Cells courtesy of Dr. Garry Nolan, Stanford University.
- Supernatant harvested and used to infect MCF10A cells.
- Select w/ G418.

Process of vector packaging



Source: http://www.stanford.edu/group/nolan/retroviral_systems/phx.html

Overview of Library Construction



Source: http://www.stanford.edu/group/nolan/screens/screens.html

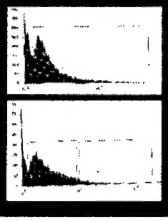
The Process of Vector Integration

- The vector is randomly integrated into the genome in regions where functional genes are found.
- The random insertion of a retroviral vector is designed to signal its presence via the activation of the reporter gene, which both mimics the expression of the endogenous gene and potentially mutates the locus.
- Endogenous gene locus is usually (but not always) inactivated by vector integration, leading to a loss of function.

Cell Sorting of Infected pool of GFP clones

- Cells sorted through flow cytometer in Dr. Fox's laboratory.
- MCF10A clones were sorted by GFP expression into positive and negative pools.
- The positive GFP expression pool was then further sorted into high, medium, and low levels of GFP expression.

Flow Cytometry Analysis



Initial sort of gene-trapped MCF-10A clones into GFP+ and GFP- pools. Sort gate 1 is the GFP- pool and sort gate 2 is the GFP+ pool.

Cell sort of gene-trapped MCF-10A clones into high, medium and low GFP expression levels. Sort gate 1 is the GFP medium pool and sort gate 2 is the GFP high pool.

Replica Plating

- Pooled clones of gene-trapped MCF10A clones plated by limiting dilution
- (1 cell/well) in 96-well plates.
- Expand in presence of G418
- Once confluent, will replica plate to 6-96 well plates per each GFP- and GFP + sort clonal cell population.
- The 6 plates will be for: basal GFP detection, master, store at – 80 degrees, 2.0 Gy , 0.5 Gy, and control.

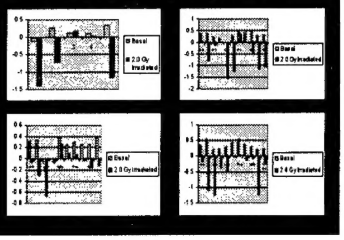
GFP expression of replica plated gene-trapped MCF-10A clones.

A. High GFP expressing MI023 gene trapped clone from replica plate.
B. Medium GFP expressing MI023 gene-trapped clone from replica plate
C. Low GFP expressing MI024 clone from replica plate

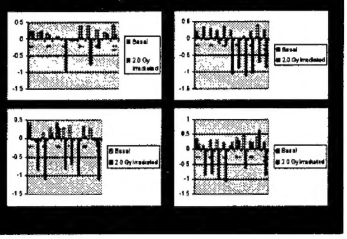
GFP detection via microplate reader

- BioRad microplate reader will be used to quantitate GFP expression.
- 96-well plates scanned at 450 nm to detect GFP by the use of TMB substrate.
- Absorbance quantitated using Microplate manager software.
- Readings taken both before & after ionizing radiation.

GFP Positive Comparison



GFP Negative Comparison



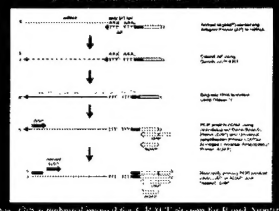
3' RACE Assay

- Rapid Amplification of cDNA ends amplifies nucleic acids sequences from a mRNA template between a defined internal site & either the 3' or 5' end of the mRNA.
- Uses natural polyA tail found in mRNA as a generic priming site for PCR.
- mRNAs converted to cDNA using reverse transcriptase and an oligo-dT adapter primer.
- cDNA amplified by PCR using a gene-specific primer that anneals to a region of known exon sequences and an adapter primer that targets the polyA tail region.

3' RACE continued

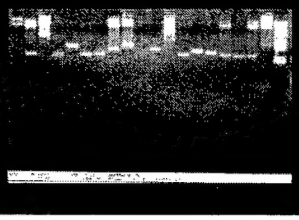
- Permits capture of unknown 3' mRNA sequences that lie between the exon and polyA tail.
- PCR products cloned into sequencing vector using Zero Blunt TOPO Cloning kit from Invitrogen.
- Nucleotide sequence of purified PCR fragments determined by sequencing with the M13 universal primers.
- Search BLASTX & BLASTN databases for homologous genes.

3' RACE Overview



Source: GIBCO technical manual for 5' RACE System for Rapid Amplification of cDNA Ends

Gel Electrophoresis: PCR of Miniprep DNA



Sequencing Results

- ???????
- 31 samples submitted to Davis Sequencing at U of C at Davis.

Future Directions

- Transformation Assays
- Repeat Assay
- Different Doses

In Summary

- HOPEFULLY, radiation-induced genes will be mapped.
- Both known & unknown radiation-induced genes will be discovered.
- Moving on to transformation assays.

Acknowledgements

- Dr. Robert Ullrich
- My committee members:
  Dr. Sue Lana, Dr. Mike Fox, and Dr. Bill Hanneman
- All members of the Ullrich lab